Cell

Engulfment Is Required for Cell Competition

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DOI 10.1016/j.cell.2007.03.054

SUMMARY

Genetic mosaics that place cells in competition within tissues may model features of tissue repair and tumor development and may reveal mechanisms of growth regulation. In one example, normal cells eliminate "Minute" cells that have reduced ribosomal protein gene dose and grow at their expense, replacing the Minute cells within developing compartments. We describe genes that are required by wild-type cells to kill Minute neighbors in *Drosophila*. The engulfment genes *draper*, *wasp*, the phosphatidylserine receptor, *mbc/dock180*, and *rac1* are needed in wild-type cells for the death of Minute neighbors, whose corpses are engulfed by wild-type cells. Wild-type cells can themselves be killed by cells with elevated engulfing activity. Thus engulfment genes act downstream of growth differences between cells to eliminate cells with reduced ribosomal gene dose.

INTRODUCTION

When two genotypes of cell are present in a chimeric individual, cell competition can favor one over the other, leading this genotype to replace the other during organ growth. Cell competition was first recognized when mitotic recombination regenerated wild-type cells in *Drosophila* heterozygous for a "Minute" mutation (Morata and Ripoll, 1975). Minute mutations represent unlinked loci encoding ribosomal proteins or other protein-synthesis components. They share a similar dominant mutant phenotype, due to haplo-insufficiency, including slow growth and reduced translational capacity. Minute heterozygotes eventually make normal-size cells and adults despite the smaller bristles that give the Minute syndrome its name (Morata and Ripoll, 1975; Boring et al., 1989; Lambertsson, 1998; Neufeld et al., 1998).

Wild-type cells are expected to grow faster than *M*/+ cells, but in mosaics wild-type clones can fill developing compartments, eliminating the *M*/+ cells that were initially in the majority (Morata and Ripoll, 1975). Wild-type clones do not cross compartment boundaries, however (Garcia-Bellido et al., 1973). The presence of *M*/+ cells accelerates the growth of wild-type cells within the same compartment, whereas the growth of *M*/+ cells is reduced when +/+ cells are present, and these interactions do not occur across compartment boundaries (Simpson, 1979; Simpson and Morata, 1981).

Cell competition also occurs between cells differing in their dosage of the *myc* gene, and in this case cells that express more Myc become "supercompetitors" that outcompete even wild-type cells (de la Cova et al., 2004; Moreno and Basler, 2004). Cells that are mutated for the "Warts" pathway of tumor suppressors (*ft*, *ex*, *hpo*, *sav*, *wts*, and *mts)* are also supercompetitors that both outgrow wild-type cells and result in their death (Tyler et al., 2007). It is thought that cells that differ in ribosome number or *myc* gene dose differ in ability to capture a limiting survival factor, such as Dpp in *Drosophila*, and that competition for Dpp leads to death of one of the genotypes at the boundary between them (Moreno et al., 2002; Moreno and Basler, 2004).

Cell competition is also thought to occur in mammals. Mouse cells heterozygous for a ribosomal protein mutant are disproportionately disadvantaged in chimeras (Oliver et al., 2004). Regenerating rat liver provides a further example, where transplanted fetal hepatocytes with high proliferative potential can replace host hepatocytes (Oertel et al., 2006).

Cell competition is thought to contribute to growth homeostasis by adjusting for variations that might occur during normal growth. Accordingly, organ size becomes more variable when competition is prevented (de la Cova et al., 2004). Cell competition is potentially important in cancer, where tumor cells might behave as supercompetitors at tumor boundaries. Consistent with this, mammalian homologs of Myc or the Warts pathway genes are associated with cancers in mammals (Nesbit et al., 1999; Edgar, 2006). Cell competition could be useful in regenerative medicine since it might prove desirable to promote the replacement of entire compartments with particular cells, without affecting other compartments.

In order to learn more about the mechanism of cell competition, we have studied the programmed cell death that occurs when *M*/+ cells share compartments with +/+ cells in *Drosophila* imaginal discs. Imaginal discs are epithelial sheets that represent primordia of adult tissues during larval life (Cohen, 1993). We found that cell death was

essential for cell competition to occur and that cell death progressively removed *M*/+ cells from the interface with +/+ regions. We found that dead *M*/+ cell corpses were engulfed by neighboring wild-type cells rather than extruded from the epithelium and consumed by macrophages. Unexpectedly, we found that corpse engulfment was not simply a passive response to the presence of dying cells but was required for cell competition to occur. Multiple engulfment genes were required specifically in wild-type cells for the killing and elimination of *M*/+ cells within the same compartment. When these genes were absent, competition was prevented, and wild-type and *M*/+ cells coexisted within the same compartment, apparently independently. Conversely, ectopic activity could promote death and engulfment of cells that did not differ in growth rate. These studies show that +/+ cells "eat" their way through mosaic compartments, consuming *M*/+ cells along the way.

RESULTS

Cell Death Is Required for Cell Competition

The importance of cell death was assessed using wing imaginal discs containing both wild-type and *M*/+ cells obtained through mitotic recombination in *M*/+ larvae. Clones descended from recombinant wild-type cells spread progressively through the *M*/+ tissue, eventually constituting most of each compartment (Garcia-Bellido et al., 1973). Mitotic recombination also generates *M*/*M* cells as reciprocal recombinants to the wild-type cells. The *M*/*M* genotype is cell lethal, and such cells were not normally seen in these experiments. Death of *M*/*M* cells is not a feature of cell competition because it is not influenced by the genotype of neighboring cells.

If death of *M*/+ cells was required to eliminate *M*/+ cells during cell competition, *M*/+ cells should persist when cell death is prevented. To test this, competition was compared in the presence and absence of the antiapoptotic baculovirus protein p35 using enGal4 to target p35 expression to posterior compartments only (Hay et al., 1994; Neufeld et al., 1998). *M(3)96C* was used in these experiments. Figure 1A shows that p35 prevented the elimination of *M(3)96C*/+ cells from posterior compartments, in accord with previous observations (Moreno et al., 2002).

Accelerated growth of wild-type regions compensates for *M*/+ cell loss (Simpson and Morata, 1981). The size of individual +/+ clones was measured 60 hr after induction (Figure 1B). Wild-type clones grew more rapidly in *M(3)96C*/+ compartments than in a nonmosaic background, but this was prevented by p35 expression. Clearly, *M*/+ cell death was essential to accelerate +/+ cell growth. The mechanism is distinct from compensation for cell death induced by other means, which is not sensitive to p35 (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004). A contribution of competitive cell death to the growth of cells overexpressing Myc has also been reported (Moreno and Basler, 2004).

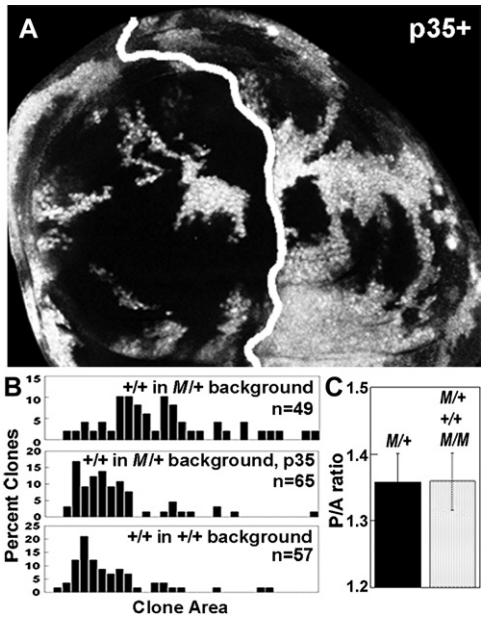

Figure 1. Cell Death Is Required for Cell Competition

(A) Third instar wing imaginal disc expressing Baculovirus p35 in the posterior compartment (right). +/+ cell clones are unlabeled (black); *M(3)96C*/+ cells are labeled for β-galactosidase expression; and the white line marks the compartment boundary. p35 expression in the posterior compartment increases the contribution of *M*/+ cells and decreases the contribution of +/+ cells.

(B) Areas of +/+ cell clones were measured and compared in different genetic environments. Clone sizes were measured 60 hr after 10 min heat shock, conditions that produce isolated, nonconfluent clones. +/+ clones grew larger in *M*/+ compartments, so long as p35 was not expressed ($p < 0.001$). p35 abolished growth stimulation ($p = 0.606$).

(C) Ratios of posterior-to-anterior wing sizes were compared. In mosaics expressing p35 like those described in (B), posterior compartment size resembled that of nonmosaic *M*/+ flies and p35-expressing flies. The areas of adult wing between L1–L3 (anterior) and L4–L6 (posterior) were measured; the vein L3–L4 region that contains parts of both compartments was excluded. $n_{m/+} = 15$, $n_{m/+,\ +/+,\ M/M} = 6$. Each posterior compartment contained about ten recombinant clones. Genotypes: (A–C) *ywhsF; en-GAL4, UAS-GFP/UAS-p35; FRT82 [armLacZ] M(3)96C/FRT82;* (B) +/+ in +/+ background*, ywhsF; en-GAL4, UAS-GFP/UAS-p35; FRT82 [armLacZ] /FRT82*.

Adult wing size is another measure of growth of +/+ cells. If growth of +/+ regions continues when death of *M*/+ cells is prevented, wings should enlarge. By contrast, if enhanced growth of +/+ clones depends on *M*/+ cell death, then mosaic wing compartments should approximate the normal size when cell death is prevented. Using anterior compartments as an internal control, we found that mosaic posterior compartments expressing p35 grew to the same size as nonmosaic ones, confirming that apoptosis of *M*/+ cells was required to enhance wild-type clone growth (Figure 1C). In these experiments, *M(3)96C*/+ wings contained an average of ten +/+ clones. These findings emphasize the central importance of cell death to cell competition.

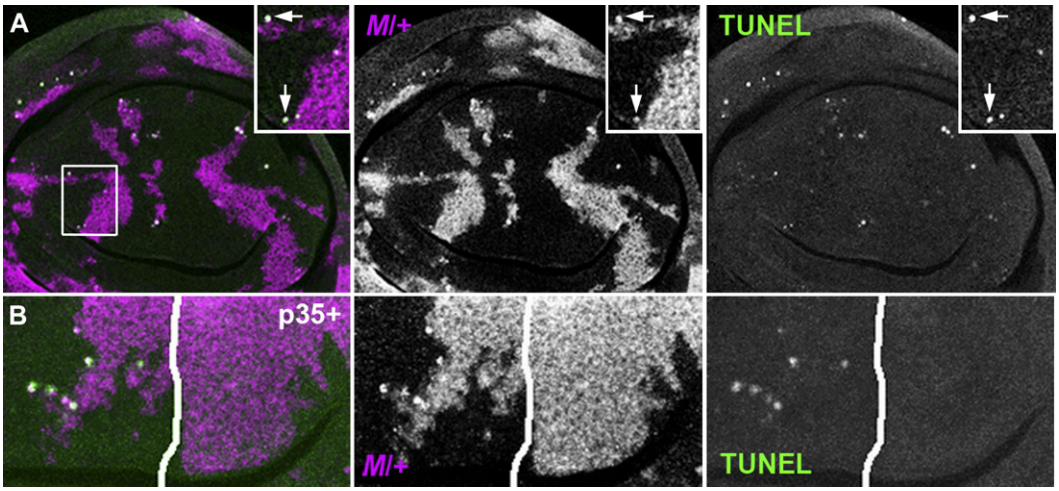

Figure 2. Cell Death Occurs at Boundaries between *M*/+ and Wild-Type Territories

+/+ cells are unlabeled. TUNEL labeling in green.

(A) Almost all TUNEL-labeled cells are *M*/+. Most are adjacent to +/+ cells (insert shows enlarged region). Cells that are most highly exposed to +/+ cells are often dying (arrows in insets). See text for quantitation.

(B) TUNEL labeling is prevented by p35 expression in posterior compartment (right). Compartment boundary traced by white line.

Genotypes: (A) *ywhsF; FRT42 [armLacZ] M(2)56F/FRT42;* (B) *ywhsF; en-GAL4, UAS-GFP/UAS-p35; FRT82 [armLacZ] M(3)96C/FRT82*.

M/+ Cells Die at Population Boundaries

The location of cell death during competition was assessed as +/+ clones spread through *M*/+ territory. Depending on the time since recombination, large interfaces between wild-type and *M*/+ cells could be examined, in contrast to the situation when *M*/+ cells were induced in wild-type compartments and rapidly eliminated from them.

Apoptotic cells were largely restricted to boundaries between wild-type and *M*/+ cell populations and were almost always of the *M*/+ genotype (Figure 2). Similar results were obtained using *M(2)56F*, which affects the gene for ribosomal protein RpS18; using *M(3)67C*, which affects RpS17; using *M(2)24F*, which affects RpL27A; or using *M(3)96C* (Figure 2 and data not shown). In the case of 361 TUNEL-positive cells in discs mosaic for *M(2)56F*, 94% were *RpS18*/+, and 71% were *RpS18*/+ cells next to wild-type cells. Amongst such *RpS18*/+ boundary cells, death was further related to the degree of exposure to wild-type cells. Only a small minority of the *RpS18*/+ cells were largely or entirely surrounded by wild-type cells, but 54% of these were dying (Figure 2A, arrows). For *M(3)96C*, 82% of *M(3)96C*/+ cells were positive for activated caspases when surrounded by +/+ cells compared with 6.2% of *M(3)96C*/+ cells at the interface with wild-type territories and 1.5% of *M(3)96C*/+ cells entirely surrounded by other *M(3)96C*/+ cells. By comparison, the frequency of apoptotic cells in nonmosaic *M(3)96C*/+ wing discs was 1.9% and in nonmosaic wild-type discs was 0.3%. These results argue strongly that proximity to wild-type cells promotes apoptosis of *M*/+ cells. The data are consistent with direct contact being responsible, as even *M(3)96C*/+ cells 1–3 cell diameters distant from +/+ cells were protected (1.4% apoptosis). These data do not exclude diffusible interactions of very short range, however. All cell death was prevented by p35 expression (Figure 2B).

The Fate of Dead Cells

Apoptotic cells are extruded during *Drosophila* embryogenesis and consumed by macrophage (Abrams et al., 1993; Tepass et al., 1994; Franc et al., 1999a). Extrusion of apoptotic cells maintains epithelial integrity while cell death occurs (Rosenblatt et al., 2001). However, dead cells can also be engulfed by neighboring imaginal disc cells, rather than by macrophage (Fristrom, 1969).

The fate of dead cells was characterized in more detail during cell competition. Dying cells were seen throughout the apical-basal axis of the epithelium but were more often basal (Figure 3). We did not observe dead cells extruded beneath the basal epithelial surface. Macrophages were rarely present in the wing disc, were most commonly basal to the epithelium where the wing disc folds, and were never found in contact with any dead *M*/+ cells (data not shown). However, we noticed cases where apoptotic material was present within neighboring, wild-type epithelial cells (Figure 3B). During interphase, the small diameter and intertwined arrangement of imaginal disc cells made it difficult to identify internalized material by light microscopy. Internalized material was clear in mitotic cells, which round up and locate apically within the epithelium (Figures 3C–3D and S1). The mitotic nature of the rounded apical cells was confirmed by labeling with an antibody against phospho-histone 3, which is specific for mitotic chromatin (Figure 3A). These data indicate that some apoptotic cells are cleared by engulfment into neighboring epithelial cells.

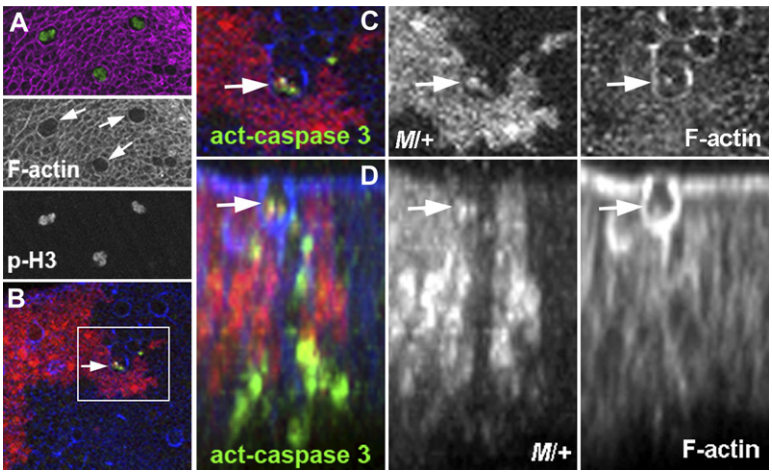

Figure 3. Engulfed Material in Mitotic Cells

(A) Apical surface of wing disc, labeled for F-actin (magenta) and mitosis (anti-pH3; green). Mitotic cells are round and predominantly apical (arrows). (B)–(D) reconstruct a mosaic wing disc to highlight a particular mitotic cell (arrows). F-actin labeling in blue. *M*/+ cells are red. Dying cells are green (CM1 antibody). (B) Apical surface of the wing disc. A mitotic +/+ cell is indicated (arrow) adjacent to the *M*/+ territory. This cell contains caspase-active material labeled as genetically *M*/+.

(C) Enlarged view and separated channels.

(D) Cross-section through the same preparation. Apical is uppermost, and basal is toward the bottom. Note that *M*/+ material is entirely within the mitotic +/+ cell (arrow), surrounded by an accumulation of F-actin, as previously reported during engulfment in *C. elegans* (Kinchen et al., 2005). Relation of other apoptotic material (green) to the majority of interphase cells is unresolved.

Genotypes: (A) wild-type; (B–D) *ywhsF; FRT42 [armLacZ] M(2)56F/FRT42*.

To identify engulfed material in interphase cells, +/+ cells were positively marked by GFP expression in mosaics where *M*/+ cells expressed β-galactosidase. In these experiments, 84% of 176 *M*/+ dead cell corpses that were surrounded by +/+ cells were colabeled with GFP as well as β-galactosidase (Figures 4A–4B, arrows). As expected if the *M*/+ material is engulfed and fuses with lysosomes, we detected GFP within the engulfed *M*/+ material but never detected β-galactosidase released into the engulfing cell. Thus these apoptotic bodies were inside wild-type cells, not in between them.

In the case of dying *M*/+ cells at the boundary with +/+ cells but not surrounded by them, individual confocal sections revealed that 11% of such cells were actually separated from other *M*/+ cells (Figure 4C). Sixty-eight percent of the boundary cells showed intermediate levels of GFP, higher than other *M*/+ cells but lower than +/+ cells (Figure 4D). These may represent early steps in engulfment. GFP was not clearly detected in the remaining 21% of dying *M*/+ cells in contact with +/+ cells, but some such GFP-negative cells nevertheless appeared morphologically as though they were being engulfed (Figures 4A–4C). Others may indeed be outside other cells or might have been engulfed by viable *M*/+ cells, which do not express GFP in this experiment. Taken together, the data show that a significant proportion of dead *M*/+ cells are engulfed by interphase +/+ cells within the wing disc epithelium, not basally extruded and phagocytosed by macrophage. *M*/+ material sometimes seen within mitotic +/+ cells may have been engulfed earlier in the cell cycle (Figure 3). Published images suggest that *M*/+ cells are also engulfed when small *M*/+ clones are introduced into a wild-type background (see Figure 3C of Tyler et al., 2007).

In cell competition between cells with different Myc levels, we also observed cells that overexpress Myc engulf adjacent apoptotic wild-type cells (Figure 4E).

When cell death was prevented by p35 expression, the isolated, engulfed cells were no longer observed (Figures 4F and 4G). This indicates that engulfment required caspase activation and death of *M*/+ cells.

Engulfment Genes Are Required for Cell Competition

In order to determine the extent of corpse engulfment in another way, we sought to prevent engulfment genetically. If engulfment was a response to the presence of apoptotic corpses, then preventing engulfment should lead to the accumulation of corpses, as is often seen in *C. elegans* (Reddien and Horvitz, 2004).

Although it was not known what genes would be required for corpse engulfment by imaginal disc cells, several genes are known to be required for phagocytosis by macrophages and glia, or by cells in other organisms. Possible roles in cell competition were explored for several homologs of genes involved in corpse engulfment in *C. elegans* or by mammalian macrophages.

One such gene was *draper* (*drpr*). *draper* encodes a transmembrane receptor that is related to the *C. elegans* engulfment receptor CED-1 (Zhou et al., 2001; Freeman et al., 2003) and that is required for phagocytosis by cultured *Drosophila* cells (Manaka et al., 2004). In addition, *drpr* has recently been found to be required in glial cells for their engulfment of fragmented axons during Wallerian degeneration and in particular instances of axon remodeling during development (Awasaki et al., 2006; MacDonald et al., 2006). We examined clones of cells homozygous for loss-of-function mutation, $drpr^{\Delta 5}$, competing with *M*/+ cells that were heterozygous for $drpr^{\Delta 5}$. If $drpr^{\Delta 5}$ homozygous cells could not engulf *M*/+ cells, we expected that

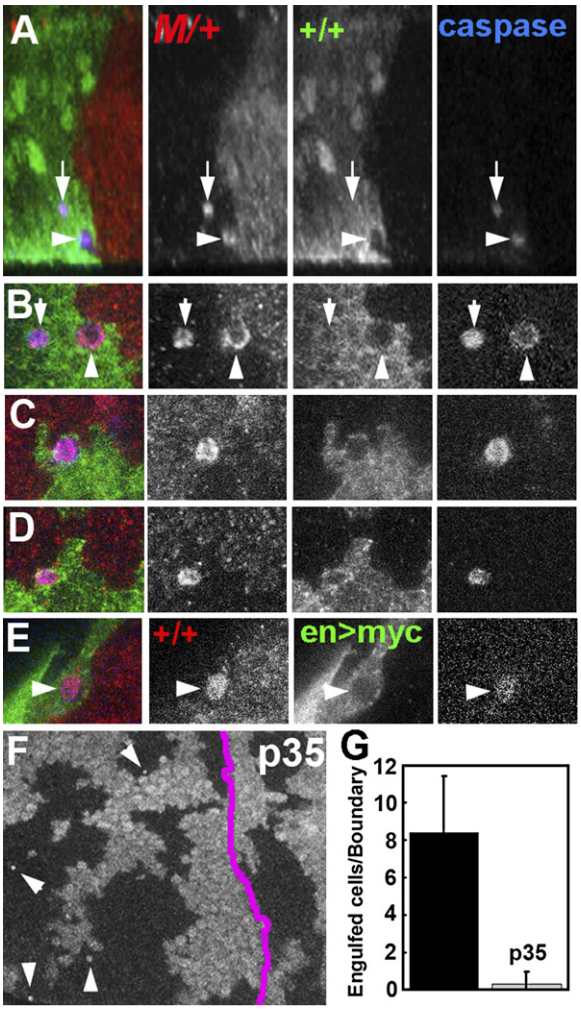

Figure 4. Engulfed Material in Interphase Cells

(A) Cross-sections of a mosaic wing disc. Apical surface uppermost. +/+ cells are green (anti-GFP). *M*/+ cells are red (anti-β-galactosidase). Apoptotic bodies (CM1 antibody) are blue. An apoptotic *M*/+ cell within the +/+ territory is GFP positive, indicating that it is within a +/+ interphase cell (vertical arrow). Another apoptotic *M*/+ cell at the boundary does not clearly contain GFP but appears to be entering the +/+ territory, perhaps by engulfment (horizontal arrowhead). Both these apoptotic bodies are basal in the epithelium.

(B) Single Z-sections of salient regions of the same specimen, at higher resolution and twice the magnification of (A). Arrow indicates the GFP-positive engulfed *M*/+ material; arrowhead indicates the boundary cell that appears almost surrounded by GFP-positive material. Only very low levels of GFP are detected in this cell.

(C) Z-section of another mosaic wing disc. An apoptotic *M*/+ material at the boundary is actually surrounded by GFP-positive +/+ cell, and is possibly being engulfed.

(D) Z-section of another mosaic wing disc. An apoptotic *M*/+ material at the boundary shows intermediate GFP labeling, above background but lower than seen for +/+ cells.

(E) Z-section of wing disc mosaic for Myc overexpression. +/+ cells are red, and *en>myc* cells are green. Arrowhead indicates apoptotic +/+ material engulfed into the Myc-expressing region and partially labeled with GFP.

(F) Mosaic wing disc expressing baculovirus p35 in the posterior compartment (right of the magenta boundary). +/+ cells are unlabeled; *M*/+ cells are labeled. Engulfed *M*/+ bodies are abundant in the anterior (arrowheads) but absent from the posterior compartment where cell death is blocked.

(G) Quantification of engulfed cells in preparations like (F; n = 19 discs, $p < 0.001$).

Genotypes: (A–D) *ywhsF; en-GAL4, UAS-GFP; FRT82 [armLacZ] [tub-GAL80]M(3)96C/FRT82;* (E) *ywhsF; en-GAL4, UAS-GFP/UAS-myc; FRT82 [armLacZ] [tub-GAL80]/FRT82;* (F) and (G) *ywhsF; en-GAL4, UAS-GFP/UAS-p35; FRT82 [armLacZ] M(3)96C/FRT82*.

apoptotic corpses of *M*/+ cells would accumulate. Instead, an unexpected phenotype resulted (Figures 5D and 5E). Apoptosis of *M*/+ cells was so reduced near $drpr^{\Delta 5}/drpr^{\Delta 5}$ clones in comparison to *M*/+ cells near wild-type clones that *M*/+ territories were never eliminated by $drpr^{\Delta 5}$ homozygous cells (Figures 5D, 5E, 5K, and 5L). In addition, we did not see the isolated, engulfed *M*/+ corpses that competition with +/+ cells was expected to produce (Figures 5I and 5J). A small number of cell deaths persisted, and almost all *M*/+ cells were in contact with the mutant cell clone but not enveloped by it. These cells may die without being engulfed, be arrested early in the engulfment process, or be engulfed into the *M*/+ cells which are not mutant for *drpr* in this experiment. Overall, it was as though little competition was occurring, similar to the situation when p35 expression blocked competitive apoptosis. To confirm that *drpr* was required for cell competition, gene function was inhibited by RNAi. Expression of dsRNA for *drpr* reduced death and replacement of *M*/+ cells within the compartment (Figure S2). These unexpected results suggested that a CED-1-like engulfment pathway was directly required in +/+ cells for them to compete with *M*/+ cells.

Mutations in *wasp* were examined as a further test of the role of engulfment. This actin regulator is required for phagocytosis of *Staphylococcus aureus* by *Drosophila* tissue culture cells (Pearson et al., 2003). Its mammalian homolog is essential to phagocytosis by macrophages and neutrophils (Zhang et al., 1999; Lorenzi et al., 2000). We studied two independent mutations, $wasp^{EY06238}$ and $wasp^{1}$, with identical results. Clones of cells homozygous for either *wasp* mutation failed to compete with *M*/+ cells. *M*/+ cell death, engulfment, and replacement were all reduced substantially (Figures 5A, 5C, 5F, 5H, 5K, and 5L).

Phosphatidylserine is a phospholipid exposed by apoptotic cells and recognized by mammalian macrophages (Fadok et al., 1992; Martin et al., 1995; Verhoven et al., 1995; van den Eijnde et al., 1998). The molecular role played by the so-called Phosphatidylserine Receptor (PSR; Fadok et al., 2000) is uncertain. PSR now appears to be a nuclear protein that may not be generally required in engulfment (Cikala et al., 2004; Cui et al., 2004; Mitchell et al., 2006), although PSR homologs are reported to contribute to corpse engulfment in *C. elegans* and in zebrafish (Li et al., 2003; Wang et al., 2003; Hong et al., 2004). We studied cells of two independent alleles, $psr^{EY07193}$ and psr^{null}, with identical results. Clones of cells homozygous

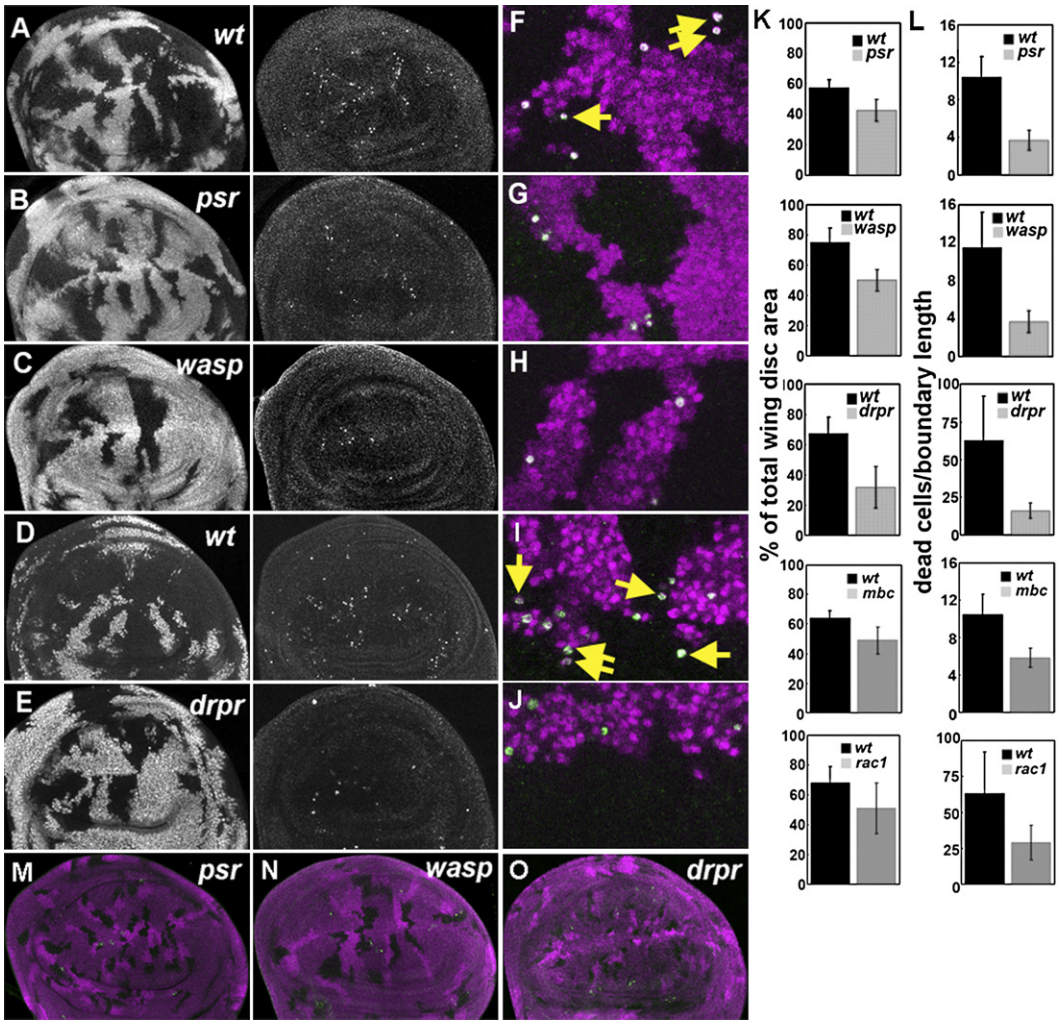

Figure 5. Engulfment Genes Required for Cell Competition

In panels (A)–(E), *M*/+ cells are labeled in the left panel. NonMinute cells of indicated genotypes are unlabeled. Dead cells are labeled by anti-activated-caspase 3 antibody in the right panel. Mutations of *drpr*, *psr*, and *wasp* reduce competitive cell death and interfere with the growth of nonMinute cells at the expense of *M*/+ cells so that *drpr*, *psr*, and *wasp* clones are smaller than controls.

(A) *FRT82* (control for psr and wasp).

(B) *FRT82psr*^{EY07193}.

(C) *FRT82wasp*^{EY06238}.

(D) *FRT80* (control for *drpr*).

(E) *drpr*$^{\Delta 5}$ *FRT80*.

Panels (F)–(J) show higher magnification images with *M*/+ cells labeled in magenta, dead cells in green, and cells of indicated genotypes unlabeled. Apoptotic *M*/+ cells were classified as entirely surrounded by nonMinute cells, at the boundary with nonMinute cells, or entirely within the *M*/+ territory. Arrows indicate dead engulfed *M*/+ cells entirely within the nonMinute territories. The number of such cells was greatly reduced in *drpr*, *psr*, or *wasp* territories.

(F) Control for *psr* and *wasp*. Apoptotic *M*/+ cells within FRT82/FRT82 territory: 19 ± 14; at the boundary with FRT82/FRT82 cells: 30 ± 26; and within *M*/+ territory: 4 ± 3.

(G) Apoptotic *M*/+ cells within FRT82*psr*^{EY07193} territory: 1 ± 1; at the boundary with *psr* cells: 6 ± 6; and within *M*/+ territory: 1 ± 1.

(H) Apoptotic *M*/+ cells within *FRT82wasp*^{EY06238} territory: 0 ± 0; at the boundary with *wasp* cells: 4 ± 2; and within *M*/+ territory: 2 ± 1.

(I) Control for *drpr*. Apoptotic *M*/+ cells within FRT80/FRT80 territory: 25 ± 6; at the boundary with FRT80/FRT80 cells: 28 ± 7; and within *M*/+ territory: 8 ± 6.

(J) Apoptotic *M*/+ cells within drpr$^{\Delta 5}$FRT80 territory: 4 ± 2; at the boundary with *drpr* cells: 14 ± 5; and within *M*/+ territory: 13 ± 9.

(K) Clonal growth quantified as % of the wing disc occupied by nonMinute cells of indicated genotypes. Between 13 and 20 wing discs were measured for each genotype in (K) and (L). In statistical tests in (K) and (L) $p < 0.001$, except for $P_{rac1area}=0.003$.

(L) Cell death rates. Quantification is per micron of boundary between genotypes.

Panels (M)–(O) each illustrate three genotypes of cells. The initial, heterozygous genotype is magenta. Homozygous mutant cells are unlabeled. Reciprocally recombinant wild-type cells show stronger magenta staining. Apoptotic cells are green (CM1 antibody). Clones mutant for each of *drpr*, *psr*, and *wasp* grew comparably to the wild-type twin-spot control. Cell death was not elevated.

for either *psr* mutation failed to compete with *M*/+ cells. *M*/+ cell death, engulfment, and replacement were all reduced substantially (Figures 5A, 5B, 5F, 5G, 5K, and 5L).

Partial reduction in competition with *M*/+ cells was also seen when nonMinute cells that were homozygous for *mbc* or *rac1* mutations (Figures 5K and 5L). These mutations affect the *Drosophila* homolog of the *C. elegans* engulfment genes *ced-5* and *ced-10*, respectively (Nolan et al., 1998; Wu and Horvitz, 1998; Reddien and Horvitz, 2000). However, a null mutation in *scar*, another actin regulator required for phagocytosis of *S. aureus* (Pearson et al., 2003), had no effect on cell competition (data not shown).

These results indicated that engulfment pathways were required for *M*/+ cell death and competition in *Drosophila*. It was important to note that in these experiments the cells homozygous mutant for *drpr*, *wasp*, *psr*, *mbc*, or *rac1*, or those expressing dsRNA for *drpr*, were not the *M*/+ cells. To confirm that engulfment genes were required nonautonomously, clones of *M*/+ cells simultaneously homozygous for *drpr*, *wasp*, or *psr* were induced in nonMinute tissues heterozygous for *drpr*, *wasp*, or *psr*. Such clones were eliminated as efficiently as *M*/+ clones, ruling out an autonomous requirement for these genes in *M*/+ cell death or elimination (data not shown). These data confirm that engulfment pathways were required nonautonomously to compete with *M*/+ cells in the same compartment.

Cells homozygous for *psr*, *drpr*, or *wasp* might show reduced competition with *M*/+ cells if these mutations themselves affected cell growth so that the two populations of cells had similar growth rates. If this were the case, we expected that cells homozygous for *psr*, *drpr*, or *wasp* would grow less well than wild-type cells and might be competed in mosaics with wild-type cells, as is the case for *M*/+ cells. Contrary to the hypothesis, clones homozygous for each of *psr*, *drpr*, and *wasp* grew equivalently to +/+ twinspots (Figures 5M–5O). In addition, cell death remained at background levels at the boundaries of homozygous *psr*, *drpr*, or *wasp* clones (Figures 5M–5O). Because it is proposed that *M*/+ cells compete inefficiently for the growth factor Dpp (Moreno et al., 2002; Moreno and Basler, 2004), we examined whether *drpr*, *wasp*, or *psr* homozygous mutant cells were less able to respond to Dpp than heterozygous cells but found no difference in the levels of Mad phosphorylation or expression of the Salm protein, both readouts of Dpp signaling during wing development (Figure S3; de Celis et al., 1996; Tanimoto et al., 2000). We also tested whether Dpp and Wg, which are sometimes released by dying cells (Huh et al., 2004; Perez-Garijo et al., 2004; Ryoo et al., 2004), could be responsible for inducing nearby cells to engulf them, but found that *M*/+ cells did not require the *dpp* and *wg* genes to be competed (Figure S4). These findings suggested that the products of the *drpr*, *wasp*, and *psr* genes are dispensable for normal growth and Dpp signaling but are nonautonomously required in +/+ cells for the apoptosis of nearby *M*/+ cells at the boundaries between +/+ and *M*/+ cell populations.

To confirm the requirement for engulfment genes through another assay and to assess their cumulative effect over time, the contributions of genotypes to adult organs were examined. When clones of +/+ cells were induced in developing *M*/+ eyes through heat-shock-induced recombination in the second larval instar, the +/+ clones contributed much more tissue to the adult eye (Figures 6A and 6B) than when genetically identical +/+ clones were induced in a nonMinute background (Figure 6C). By contrast, clones of *psr*, *drpr*, or *wasp* homozygous cells made smaller contributions to the adult eye in *M*/+ backgrounds (Figures 6D–6F) but similar contributions to wild-type cells in nonMinute backgrounds (Figures 6C and 6G–6I). These findings confirm that the *psr*, *drpr*, and *wasp* genes are required for +/+ cells to compete with *M*/+ cells and not for the growth or survival of +/+ cells.

Expression of a Wasp Activator Promotes Death and Engulfment of Wild-Type Cells

These findings raised the question of whether differences in growth rate were always essential for cell competition or whether engulfment and death could be stimulated between cells of equal growth rates. Engulfment-signaling pathways converge on cytoskeletal rearrangement and process extension (Kinchen et al., 2005). To activate Wasp at the plasma membrane, a myristylated form of the *Drosophila* HEM protein Kette was expressed in clones of imaginal disc cells (Bogdan and Klambt, 2003). These grew equivalently to control clones expressing GFP alone and had normal Dpp signaling (Figure S3). myr-Kette expression caused apoptosis in 4% of the expressing cells (compared to 0.2% of cells expressing GFP alone) and also in 1% of neighboring wild-type cells (compared to 0.3% of wild-type cells neighboring GFP-expressing clones); survival of cells more than one cell-diameter away was not affected (Figures 7A–7B). Eighty-one percent of the dying cells adjacent to myr-Kette-expressing cells became engulfed by them (compared to 33% of dying cells engulfed into cells expressing GFP alone; Figures 7A–7B and data not shown). These results suggest that activated cells may induce engulfment

(M) *FRT82psr^{EY07193}*.
(N) *FRT82wasp^{EY06238}*.
(O) *drpr$^{\Delta 5}$FRT80*.
Genotypes: (A) and (F) *ywhsF; FRT82 [armLacZ] M(3)96C/FRT82;* (B) and (G) *ywhsF; FRT82 [armLacZ] M(3)96C /FRT82 psr^{EY07193}*; (C) and (H) *ywhsF; FRT82 [armLacZ] M(3)96C /FRT82 wasp^{EY06238}*; (D) and (I) *ywhsF; [UbiGFP] M(3)67C FRT80/FRT80*; (E) and (J) *ywhsF; [UbiGFP] M(3)67C FRT80/drpr$^{\Delta 5}$ FRT80;* (M) *ywhsF; FRT82 [armLacZ]/FRT82 psr^{EY07193};* (N) *ywhsF; FRT82 [armLacZ]/FRT82 wasp^{EY06238};* (O) *ywhsF; [armlacZ] FRT80/drpr$^{\Delta 5}$ FRT80.*
(K) and (L) include in addition:
*ywhsF; FRT82 [armLacZ] M(3)96C /FRT82 mbc ^{EY01437}*and *ywhsF; [UbiGFP] M(3)67C FRT80/rac1 ^{EY05848} FRT80.*

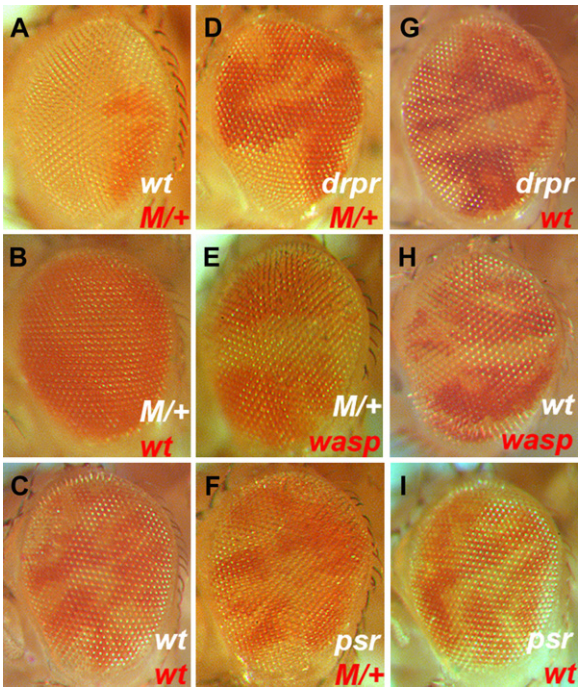

Figure 6. Engulfment Genes Are Required for Competition throughout Development

Panels (A)–(I) show eyes from adult *Drosophila* where mosaicism was induced early in development using hsFlp (A, B, D, E, or F) or eyFlp (C and G–I). Such adults illustrate the overall effects on growth throughout development.

(A) +/+ clones (white) out-compete *M*/+ (red).

(B) +/+ clones (red) out-compete *M*/+ (white).

(C) +/+ clones (white) do not compete with +/+ (red).

(D) *drpr* clones (white) do not compete with *M*/+ (red). Matched FRT82 control in (A).

(E) *wasp* clones (red) do not compete with *M*/+ (white). Matched control in (B).

(F) *psr* clones (white) do not compete with *M*/+ (red). Matched control in (A).

(G) *drpr* clones (white) are not competed by *drpr*/+ (red).

(H) *wasp* clones (red) are not competed by *wasp*/+ (white).

(I) *psr* clones (white) are not competed by *psr*/+ (red).

Genotypes: (A) *ywhsF; [UbiGFP] M(3)67C FRT80/FRT80;* (B) *ywhsF; FRT82 M(3)96C /FRT82 [armLacZ];* (C) *yweyF; FRT82 [armLacZ]/ FRT82;* (D) *ywhsF; [UbiGFP] M(3)67C FRT80/drpr$^{\Delta 5}$ FRT80;* (E) *ywhsF; FRT82 M(3)96C /FRT82 wasp^{EY06238};* (F) *ywhsF; FRT82 [armLacZ] M(3)96C/FRT82 psr^{null};* (G) *yweyF; [armlacZ] FRT80/ drpr$^{\Delta 5}$ FRT80;* (H) *yweyF; FRT82/FRT82 wasp^{EY06238};* (I) *yweyF; FRT82 [armLacZ]/ FRT82 psr^{null}*.

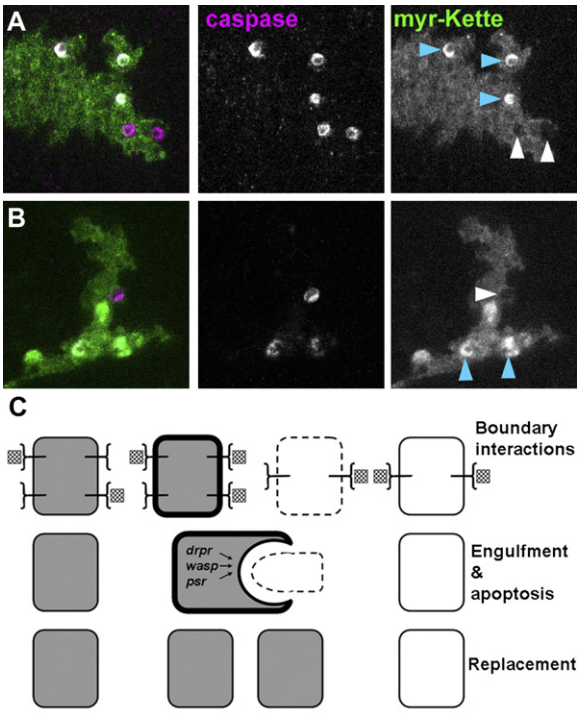

Figure 7. Role of Engulfment during Cell Competition

(A) and (B) show clones of wing disc cells coexpressing GFP and the Wasp activator, myr-Kette. Apoptotic cells are labeled with anti-activated caspase (magenta). In (A), three *myr-kette* cells are apoptotic (blue arrowheads). Their GFP contents are typically condensed (like examples in Figure 4). Two adjacent wild-type cells are also apoptotic (white arrowheads); one has been engulfed into the *myr-kette* clone and has an intermediate level of GFP. (B) shows further examples of apoptotic *myr-kette* cells (blue arrowheads) and an adjacent wild-type cell (white arrowhead) that is apoptotic and engulfed. Apoptosis is rare away from *myr-kette* clones (no examples shown) or in cells expressing GFP alone (not shown). Genotype: *ywhsF; act>CD2>GAL4, UAS-GFP/UAS-kette^{myr}*. Larvae were heat shocked at 37°C for 30 min at 60 ± 12 hr after egg laying and were dissected 60 hr later.

(C) Model for cell competition. It is thought that wild-type cells (shaded) compete with Minute cells (unshaded) for a growth factor such as Dpp (Moreno et al., 2002). Here Minute cells are shown with less receptor, one way in which ribosome gene dose might perturb a signaling pathway. Because wild-type cells at the boundary capture more factor and Minute cells capture less, the Minute cells are predisposed toward apoptosis by sublethal caspase activity, and the wild-type cells to engulf their corpses using *drpr*, *wasp*, and other genes. Both changes are required to initiate cell competition: *M*/+ cells are not killed and engulfed by other *M*/+ cells; wild-type cells at the boundary only engulf and kill *M*/+ cells and cannot engulf *M*/+ cells expressing p35. Ectopic Wasp activation can engulf and kill some +/+ cells that have no disadvantage in growth or Dpp signaling, however, so the growth differences that initially weaken *M*/+ cells may be bypassed by sufficient engulfment activity. The boundary between Minute and nonMinute populations progressively shifts as growth in induced to replace territory previously occupied by engulfed Minute cells by wild-type cells.

and apoptosis of at least some neighboring wild-type cells, even when the latter are not disadvantaged in growth.

DISCUSSION

Our main finding is that out-competed *M*/+ cells are not eliminated simply by depletion of growth and survival signaling but also by parallel induction of engulfing activity in neighboring cells. Cell competition cannot occur without engulfment, which can even cause death of wild-type cells. Our current model of cell competition is shown in Figure 7C.

Competition Kills Cells at the Competitive Boundary

We were able to study extensive boundaries between +/+ and *M*/+ territories of approximately equal size by introducing +/+ cells into *M*/+ tissues. These studies make it clear that the cell death on which cell competition depends is highly localized and almost restricted to *M*/+ cells juxtaposed to +/+ cells. Interestingly, studies of repopulating rat liver also described a wave of apoptosis at the interface where tissue replacement occurs (Oertel et al., 2006).

In cell competition, we found apoptotic corpses not only within the +/+ territory but actually within the +/+ cells. Some corpses may remain outside other cells, but we cannot distinguish such cells (should they exist) from recently engulfed corpses that are not yet labeled with GFP or from corpses engulfed by viable *M*/+ cells which lack GFP in our experiments (Figures 3 and 4). Cells that overexpress Myc also engulf nearby cells (Figure 4E), although they may kill cells at a distance in addition (de la Cova et al., 2004; our unpublished results). Engulfment distinguishes cell competition from other processes that eliminate defective cells, e.g., cells with defective Dpp signal transduction or Src kinase activity, which are extruded from the epithelium regardless of cell death (Gibson and Perrimon, 2005; Shen and Dahmann, 2005; Vidal et al., 2006).

It was a surprise that cells that were mutant for engulfment genes did not compete with, kill, or engulf *M*/+ cells. These are the first genes found to be required in +/+ cells for cell competition to occur, and they show that diminished survival signaling is not solely responsible for killing *M*/+ cells (Figures 5 and 6).

The simple view that engulfment clears apoptotic corpses that die for cell-autonomous reasons had already been complicated by results from *C. elegans*. In worms that are homozygous for a hypomorphic allele of the caspase gene *ced-3*, cell death is further reduced by loss-of-function mutations in the engulfment genes (Hoeppner et al., 2001; Reddien et al., 2001). The *C. elegans* data indicate that, under conditions where cells that are programmed to die are close to survival because of reduced caspase activity, engulfing activity can tip the scales toward apoptosis. Our results indicate an even greater role in *Drosophila* cell competition so that engulfment is necessary for cell death, and it is the cell-autonomous cell-death program that is insufficient to remove *M*/+ cells by itself (Figure 7C).

The Importance of Engulfment to Cell Competition

An extreme notion was that all imaginal disc cells might continuously strive to kill and engulf one another but normally be too evenly matched to do so. In this model, competition would occur because *M*/+ cells are less phagocytic and so are prone to engulfment and assassination by their neighbors. We reject this model because clones of homozygous *psr*, *drpr*, or *wasp* mutant cells, which have reduced engulfing activity, were not eliminated by their heterozygous neighbors (Figures 5 and 6). Because cells that are less able to engulf are not at a disadvantage, engulfment activity must be triggered at competing boundaries, not constantly present (Figure 7C).

Does engulfment activity alone determine where and when cells die? Engulfment of wild-type cells by cells where Wasp is activated by myr-Kette is consistent with this notion (Figures 7A–7B). However, wild-type cells specifically kill and engulf *M*/+ cells, not other +/+ cells. Perhaps *M*/+ cells are made targets by sublethal caspase activity, given that wild-type cells cannot engulf *M*/+ cells in which baculovirus p35 is expressed.

If weakened *M*/+ cells were sufficient to initiate cell competition, we would expect that boundary *M*/+ cells would be killed by their neighbors of both +/+ and *M*/+ genotypes. Mosaic analysis with engulfment mutations shows that any engulfment that occurs into *M*/+ cells is neither required nor sufficient to sustain cell competition in the absence of engulfing wild-type cells. All-in-all, the data suggest that both diminished survival signaling in *M*/+ cells and activated engulfment in +/+ cells occur and are necessary in parallel to kill *M*/+ cells at the boundary (Figure 7C).

Role of Cell Competition in Development

Flies null for *psr* or *drpr* or those homozygous for the $wasp^{EY06238}$ mutation are viable and resemble wild-type morphologically; this is in agreement with previous conclusions that cell competition is not essential for non-mosaics under laboratory conditions, although it does fine-tune growth regulation (de la Cova et al., 2004). In addition, cell competition may provide a selection for the best cells available to construct compartments (Baker and Belote, 1983) in which "underperforming" cells are recognized and eliminated, perhaps analogous to mechanisms by which nonself cells are phagocytosed (Franc et al., 1999b). Cell competition might prove useful for tissue repair. Manipulating engulfment could be one component of an approach to replace developing compartments and might not require cells with higher growth rates or oncogene activity than wild-type cells to achieve replacement.

EXPERIMENTAL PROCEDURES

Mosaics

Mosaic clones were obtained by the FRT-FLP technique (Xu and Rubin, 1993; Newsome et al., 2000). The MARCM technique was used to achieve labeling of both *M*/+ and +/+ cells, and of both +/+ and *en>myc* cells (Lee and Luo, 1999). Flies were maintained at 25°C. Where appropriate, 1 hr heat shock at 37°C was administered 60 ± 12 hr after egg laying for dissection 60 hr later, unless indicated otherwise. For Minute genotypes, heat shock was 84 ± 12 hr after egg laying and dissection 72 hr later, unless indicated otherwise.

Clones simultaneously homozygous for *drpr*, *wasp*, or *psr* and heterozygous Minute were obtained in the following genotypes:

M(1)Bld/ywhsF; FRT82 [armLacZ] [RpL36+] / FRT82 $psr^{EY07193}$.
M(1)Bld/ywhsF; FRT82 [armLacZ] [RpL36+] / FRT82 $wasp^{EY06238}$.
M(1)Bld/ywhsF; [armLacZ] [RpL36+] FRT80 / $drpr^{\Delta 5}$ FRT80.

Other genotypes are described in the figure legends. See Supplemental Data for a full listing of mutations and transgenic strains.

Immunohistochemistry

Antibody and TUNEL labeling were performed as described (Firth et al., 2006). F-actin was labeled by Phalloidin-546 (Molecular Probes). See Supplemental Data for details of antibodies used. Images were recorded using a BioRad Radiance 2000 confocal microscope and processed with ImageJ and Adobe Photoshop. Sequential imaging mode was used for the GFP/LacZ/caspase analysis shown in Figure 4.

Pooled data are represented as mean ± 1 standard deviation. Statistical significance was determined by Student's t test.

Supplemental Data

Supplemental Data include Experimental Procedures, References, and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/6/1215/DC1/.

ACKNOWLEDGMENTS

We thank L. Johnston, D. Shafritz, and Kristin White for discussions and comments on the manuscript and C. de la Cova for suggesting two-color engulfment assays. We thank Marc Freeman, C. Klambt, G. Morata, K. White, E. Schejter, R. Eisenman, and J. Treisman for *Drosophila* strains and M. Freeman and K. White for sharing unpublished information and reagents. Some other strains were obtained from the National *Drosophila* Stock Center at Bloomington, Indiana. Confocal microscopy was performed at the AIF, AECOM. This work was supported by a grant from the NIH (GM61230). N.E.B. is a scholar of the Irma T. Hirschl Trust for Biomedical Research. Some data in this paper are from a thesis to be submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the Graduate Division of Biomedical Sciences, Albert Einstein College of Medicine, Yeshiva University, USA.

Received: October 23, 2006
Revised: January 22, 2007
Accepted: March 29, 2007
Published: June 14, 2007

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